Appendix 1

TABLE 1: Product Characterization and Manufacturing Process Deficiencies and Shortcomings.

OCSPP Guideline	MRID	Result	Data or information needed
880.1100 *Product identity and composition*	506987-01	**Deficient**	• **Penetrance**: The degree of penetrance of the female-lethal trait has been evaluated in backcrosses of OX5034 with its corresponding Latin WT strain background. No live females were reported in the progeny of these matings and thus penetrance was reported to be 100%. **Having no live female mosquitoes will be a critical component of the risk assessment; however, more data is needed to support this for environmental releases. Demonstrate that the degree of penetrance of the lethal trait remains stable at 100% in other genetic backgrounds of *Ae. aegypti*, such as those found in Texas and Florida.** • **Latin wildtype strain**: Elaborate on the history and biology of the Latin wildtype (LWT) strain. Does it have any strain-specific traits that are relevant to human health? How long has this strain been maintained under laboratory conditions, including the time prior to acquisition by Oxitec in 2006? Do you know of the purpose of maintaining this strain at INSP, MX, e.g., was it originally sampled due to a specific trait? • **Manufacturing process**: The manufacturing process must be provided as a standalone study (receive its own MRID) and describe the initial transformation process in greater detail than currently presented. For example, identify the reagents that were used for transformation and how the zygosity of the transformants and the penetrance of the trait were determined. With respect to the referenced studies, i.e., Morris, 1997 and Jasinskiene *et al.*, 1998, please indicate the relevant parts for manufacture of OX5034 and note any deviations. The manufacturing process must also describe the protocol that will be used to introduce the OX5034 traits into a new WT strain, in the event that resistance through assortative mating occurs, as well as any QC procedures, such as determination of complete penetrance. If introgression of the trait will follow the existing manufacturing process, you may simply refer to that process in your protocol. Relatedly, have you ever introduced genetic diversity into the OX5034 line as part of the regular manufacturing process? • **QD-SOP-00033 "Change control process"**: Note that once a pesticide manufacturing protocol has been approved by EPA, the developer is bound to that procedure and any subsequent changes have to be reviewed by the Agency before they may be implemented. Thus, protocol QD-SOP-00033 and associated references must be removed from the SOPs.

			• **Definition of a "batch"**: How do you define a "batch" for releases of eggs, pupae, and adults, e.g., 2.2.5 and 2.3. Is it possible that a single batch could be divided into different developmental stages for field release? • ***Aeadsx* splicing module**: Based on the literature (e.g., Salvemini *et al.*, 2011) and your description of the OX5034 *Aeadsx* splicing module, it appears possible that all three splice variants of the gene (F1, F2, and M) are translated and thus present as protein isoforms in OX5034. Please substantiate your hypothesis that $Aeadsx^{F1}$ and $Aeadsx^{M}$ will enter the nonsense-mediated decay pathway and that $Aeadsx^{F2}$ (as part of the tTAV variant) is the only proteinaceous isoform expressed in any of the OX5034 lifestages. • **rDNA insertion site:** Elaborate on how comparison of the genomic flanking sequences obtained from SuperContig1.420 with the sequences deposited in the two referenced transcript databases informs whether expression of the two genes AAEL009706 and AAEL018077 are altered in OX5034 compared to the LWT. • **Sanger sequencing of OX5034 rDNA insertion**: Provide an annotated OX5034 rDNA sequence (Fig. 9), that identifies at a minimum the inert and active ingredient sequences and annotations for each *Aeadsx* splicing module intron and exon. Further, provide the native *Aeadsx* sequence and annotate the changes made to exon 5b that allowed for the creation of the ORF. • **Confirmation of sex-splicing of the tTAV gene**: $Aeadsx^{F1}$ expression in OX5034 and LWT controls must be presented (Fig. 5). • **OX5034 longevity:** Please provide data on the longevity of homozygous and hemizygous OX5034 males (reared without Dox) and females (reared with Dox) in the lab. • **Annual field testing for presence of OX5034 females (2.5.1.)**: An acceptance threshold of 85% trait penetrance is proposed. Define "penetrance" in this context, i.e., is this equivalent to 85% lethality in female progeny? Provide a justification for this threshold and the proposed annual testing interval. What are the analytical and sampling methods you propose that will ensure that any OX5034 females will be detected in the environment? Include these measures into your experimental protocol and provide instructions on mitigation measures in case female OX5034 individuals are identified. You propose that molecular techniques, such as PCR may also be useful for identification of the OX5034 construct (2.4. Safety Assessment). Please include these methods of detection into your SOPs to be used concurrently with the fluorescence-based screening.
Testing for presence of viral infections	506987-01 *Appendix 1*	**Deficient**	• Must be provided as a standalone study (receive its own MRID). • Provide a rationale for the annual testing interval. • Testing for the presence of the Zika virus must be included in the protocol. • Hinson *et al.*, 2015 (cited on the VecTOR® test systems, Inc. website) indicate that increasing the development of the VecTOR® wicking assay can significantly reduce false negative results. Specifically, the authors conducted an assay in which a single known CHIKV-infected mosquito was tested in a pool of up to 50 mosquitoes. At first, the test strips were dried for 20 min (as outlined in QD-R-00087 and per manufacturer instructions), resulting in only ≥60% of the test

			strips identifying the presence of the infection. By extending the incubation period to 1-hour, positive test results increased to the expected 100%. Please address the concerns raised by the publication. • The SOP must include instructions on how to avoid deployment of a mosquito batch in which a viral infection has been detected and these measures must be captured in the same study.
Investigating the self-limiting phenotype and penetrance	506987-17	**Deficient**	• **Clutch size**: Please elaborate on how the clutch sizes determined in this study compare to the clutch size of other *Ae. aegypti* strains from areas in which field releases are planned. • **Phenotype and penetrance assay** (page 8; 5.4): It says that the tested strain has been maintained in the lab for 15 generations (homozygous for 5 generations; study completed Feb 22, 2019). Given that the OX5034 strain has been described elsewhere (MRID 50698701, page 33) as an insect colony equivalent of over 31 generations (as of January 04, 2019), please clarify whether the strain used for the study is equivalent to the one for which the EUP is sought.
Evaluation of insect susceptibility status	506987-18	**Sufficient for review**	• Please clarify whether "OX5034O" and "OX5034" denote the same strain for all studies submitted in support of this application.
Quantitative Detection of DsRed2 and tTAV protein in whole body extracts of OX5034	506987-19	**Deficient Unless 100% Penetrance of the Female-Lethal Trait Supports Environmental Release**	• rtTAV concentration must be empirically determined by comparing the rtTAV amount against BSA for a given expression batch (9. Appendix B). It appears that concentration is currently based on visual approximation only. • Explain how the protein bands were normalized, e.g., band intensity, band size, software. • Confirm the identity of the tTAV protein used as a loading standard, i.e., is this the tTAV as expressed in OX513A or OX5034. • Provide an explanation for the presence of a protein band at the molecular weight of rDsRed2 in the homozygous LWT pupae (rep 2, page 43). • Please see comment on study 506987-02 regarding the relevancy of the tTAV and DsRed2 proteins as expressed in OX513A.
Bioinformatics analyses & literature search	506987-20	**Deficient Unless 100% Penetrance of the Female-Lethal Trait Supports Environmental Release**	• **DsRed2 cytotoxicity**: The literature provides several examples that indicate that fluorescent proteins, including DsRed2, can exert a certain level of cytotoxicity. One such example is Hadjantonakis *et al.*, 2002, referenced in Ryu *et al.*, 2013 (MRID 506987-20), in which the authors note that: "*Mutant DsRed transgenic mice have not been popular because it was believed to be difficult to obtain widespread expression of DsRed due to its cytotoxic effects (Hadjantonakis et al., 2002).*" The presented study fails to uncover that information. However, it is important that it be added and its relevance to the pesticidal product discussed to provide a comprehensive picture of the potential for DsRed2 to cause adverse effects to humans and other non-target organisms.
Protein digestion & permeability - Bioinformatics	506987-21	**Deficient Unless 100% Penetrance of the Female-Lethal Trait Supports**	• See comment on study 506987-02 regarding the acceptability of study 504651-14. • Please correct the DsRed2 MW weight and charge calculations.

		Environmental Release	
SOPs for production of OX5034	506987-23	**Deficient**	• **Production facilities:** Identify all OX5034 production facilities (physical addresses), their biological containment level, and which manufacturing process(es) they will adhere to for the duration of this EUP.

TABLE 2: Mammalian Toxicity Deficiencies and Shortcomings.

OCSPP Guideline	MRID	Result	Data or information needed
870.1100 *Acute oral toxicity*	506987-02	**Deficient Unless 100% Penetrance of the Female-Lethal Trait Supports Environmental Release**	• The scientific rationale to waive the requirements for mammalian testing relies in part on the *in vitro* protein digestibility study conducted in support of OX513A (MRID 504651-14). That study does not demonstrate protein lability of either DsRed2 or tTAV in part due to the absence of visible protease bands in the Coomassie-stained gels and the small amount of rtTAV starting material. **Furthermore, OX513A expresses proteins that are different from those present in OX5034. Therefore, we suggested that you provide additional information to substantiate your claim that neither the active, nor the inert protein pose adverse effects to humans, e.g., a new *in vitro* protein digestibility study that is specific to the OX5034 active and inert ingredients.** • Similar to the request for MRID 506987-20, please discuss the literature on the potential cytotoxic effects of DsRed2. • The waiver rationale is based on lack of allergenicity. Given that DsRed2 was initially found to share significant homologies to the putative allergen GFP-like protein Akane (U.S. EPA, 2018; Kato *et al.*, 2017), please provide or cite any information you may have from the AllergenOnline and COMPARE databases regarding their decision to remove the GFP-like protein Akane from their list of putative allergens.
870.1300 *Acute inhalation toxicity*	506987-03	**Deficient Unless 100% Penetrance of the Female-Lethal Trait Supports Environmental Release**	• Same deficiencies as for OCSPP Guideline 870.1100.

870.2400 *Primary eye irritation*	506987-04	**Deficient Unless 100% Penetrance of the Female-Lethal Trait Supports Environmental Release**	• Same deficiencies as above for OCSPP Guideline 870.1100.
870.2600 *Primary dermal irritation*	506987-05	**Deficient Unless 100% Penetrance of the Female-Lethal Trait Supports Environmental Release**	• The study does not address the potential for DsRed2 and tTAV to be hazardous, but in turn solely relies on the assumption that exposure to these proteins is negligible. A discussion of the potential hazard must be provided, including toxicity and allergenicity.

Appendix 2

Names for ingredients must have the vector included in the name, see below:

- *Active ingredient:* Tetracycline repressible Transactivator Protein Variant (tTAV-OX5034) protein and the genetic material (vector pOX5034) necessary to produce the protein *in vivo*
- *Inert ingredient*: DsRed2-OX5034 protein and the genetic material (vector pOX5034) necessary to produce the protein *in vivo*

Appendix 3

Table 1: Nontarget organisms and environmental fate deficiencies and shortcomings.

OCSPP Guideline	MRID	Result	Data or information needed
850.1010 (Daphnia replacement) *Aedes aegypti strain OX5034 larvae (batch RD021018): 96 hour feeding study with the American (Signal) crayfish*	506987-07	**Sufficient for review**	• What is the reasoning for using untreated diet as the control and Latin WT larvae as the reference? In the *Poecilia* study, Latin WT larvae were used as the control and a positive control (potassium dichromate) was used as the reference. • What is the age of the crayfish used in the study? Given the background information provided (pg. 8), the juvenile stage is likely the most relevant.
850.1075 *A laboratory toxicity study […] Poecilia reticulata (Actinopterygii: Poecilidae) under semi-static conditions*	506987-08	**Sufficient for review**	• What is the age of the fish used in the study?
850.2100, 850.2200, 880.4350 *Request for waiver from avian testing*	506987-09 506987-10	**Sufficient for review**	• Remove the reference to rapid digestion of the proteins by gastric enzymes. The cited study MRID 504651-14 is not relevant in this context. • The MRIDs state: "*Aedes aegypti* are primarily found in urban areas and have little or no interaction with avian species in natural ecosystems". Urban areas are part of the larger ecosystem and there are nontarget species, including avian species,

			present. Revise this sentence and related points.
850.2400 *Wild mammal toxicity testing*	-	**Not submitted**	• Toxicity testing or waiver rationale should be submitted for wild mammals given that mosquitoes are a known food source for bats.
880.4350 *Request for waiver from nontarget insect testing*	506987-13	**Sufficient for EUP Review. Data Needed for Registration that Could be Generated During EUP**	• Introgression of other traits besides insecticide susceptibility (pg 11-12; 4.3). How will Oxitec evaluate whether invasive traits (e.g., increased fecundity) have evolved in OX5034 mosquitoes due to lab rearing conditions (Leftwich *et al.*, 2016)? If such a trait is present, how will Oxitec monitor whether it is introgressed into the wild population during releases? • The MRIDs states: "*Aedes aegypti* are primarily found in urban areas and have little or no interaction with nontarget insects in natural ecosystems". Urban areas are part of the larger ecosystem and there are nontarget insect species present. Revise this sentence and related points.
Analysis of no effect to threatened or endangered species or critical habitat	506987-14	**Sufficient for review**	• OX5034 longevity (pg 12; first bullet): Oxitec mentions that released OX5034 males only survive a few days. However, eggs and pupae will also be released. It is therefore useful to know if the time of adult stage of OX5034 males in the field lengthens when released at earlier life stages.